Name of Mouse model or mutation:
MMACHC-FLOX-EM1-B6

Description:
Floxed allele made by CRISPR/Cas9 gene editing.

Type of mutation:
Floxed allele: Mmachc-201 Exon3: ENSMUSE00000181372

Delivery method:
Pronuclear injection into 1-cell stage embryo

Genetic Background:
C57BL/6J

Nuclease:
Cas9 mRNA

sgRNAs:

Protospacer sequence	PAM sequence
CTCAATAACTCCTATAATGG	AGG
AGGAGTTATTGAGTTGTCTG	TGG
GGAATGAGTGTGCACTGAGT	GGG
GCACACTCATTCCATGCAGA	AGG

lssDNA donor sequence (5'-3'):

LOCUS Tm1c 1444 bp DNA linear 23-OCT-2020
FEATURES Location/Qualifiers
misc_feature 228..261
/note="loxP"
misc_feature 1144..1177
/note="loxP"
misc_feature 220..227
/note="AsiSI (SfaAI)"
misc_feature 1178..1185
/note="MreI"
PCR_primer 199..219
/note="LoxPF"
PCR_primer complement(1186..1205)

```
                /note="LoxPR"
     misc_feature    1206..1444
                /note="3'HA"
     misc_feature    1..198
                /note="5'HA”
     misc_feature    262..1143
                /note="Critical Region (protected)"
     misc_feature    634..787
                /note="Mmachc-201 exon3: ENSMUSE00000181372"
     source          1..1444
                /dnas_title="Tm1c gBlock template Mmachc_Flox_revised"
ORIGIN
        1 TCTAGTCTTA ATCTTAAAGA TGTTGGAATA TTAGTAATCC AACATCTTTA GTCATTGGTC
       61 ATTGACTTTA GTCATTGGGT TCTATGCTGT CTGTGGAGCC TTTGAAGGCA GAAGACTATT
      121 TCCCAGCCTG TACTCATTTA TATACACATA AATCTTTACA TATACATTCT AGGTCTTTCC
      181 ACTCTAGAAT TGCCACAGat ccgggggtac cgcgtcgagG CGATCGCATA ACTTCGTATA
      241 GCATACATTA TACGAAGTTA TTGGAGGCTT CTGAGCAAGG CTTTTATATT TCCTTTCAAG
      301 CACAGGCCCT TCTGAGGGGG CAGAGAAACT CTAAAATCCT TCACTTTGTA GCTCCTTAGG
      361 TTAAAGTTGT TTTTCCTTCA AACAGACATT TAATGGTGAA TTCCAGCAGT AGAGAGATAG
      421 AGGCAGGAGA ATCAGGAATT CATGATCATC TTTAGATATA TAAAGCATGT TCAAGGTCAT
      481 TCTGACCCGG TCACACACAC ACACAAACAA ACAAACAAAC AAAAAAATCC CCTTGGGCTT
      541 ATAAAAACAG AAGGCATATT AATACATAAC TTAGGACTTA GAACAAAATC ATGACTCCCA
      601 TCATGCTAAC AAGCGCCCTG TATTCTGTTC CAGAAGTTTC CAGAAGTGCA TATGGAAGTC
      661 ATTGCTGACT ATGAGGTACA CCCCAATCGG CGACCTAAGA TTCTCGCCCA GACAGCAGCC
      721 CATGTGGCAG GTGCTGCTTA TTACTACCAA CGACAAGATG TGGATGCAGA CCCATGGGGG
      781 ACCCAGGTTA GAGAGTGAAT GTGAATGAGT GGGTGGGGAC CAAGGGAAAG GATAGTAAAG
      841 GCTCCTAAAG GATCCCCTAC AATATAAGGT CTGTATAAGT GCTGAGATTA AAGGTGTGCC
      901 CCACCACTGC CCGGCTAGAT TTTTTTTTTA AAGATTTATT TATTTATTGT ATGTAAGTAC
      961 ACTGTAGCTA TCTTCAGACA CACCAGAAGA GGGCATCAGT TCTCGTTACA GATGGTTGTG
     1021 AGCCACCATG TGGTTGCTGG GATTTGAACT CAGGACCTTC AGAAGAGCAG TCAGTGCTCT
     1081 TCACCACTGA GCCATCTCTC CAGCCCTAGA TTTCTTAATT TAATAGAAGG GACAGAGCCT
     1141 TCTATAACTT CGTATAGCAT ACATTATACG AAGTTATCGC CGGCGggtct gagctcgcca
     1201 tcagtAGTGG GAAAGAATTA GAAAAAGATT AGGAACATTT GTTGCTTTTG TATGAACCAT
     1261 GTTCAATCCC AAGCACTCAC GTGGCTGCTC AGAACCATCT GCAACTCGTT TTTAGATTGA
     1321 CACCCTCTGC TGGCCGCTGT GGGTACTGCT TGCATGCAAG CAAAACACTC ATACCCATAC
     1381 TTGGGGGATA AAAAGTTTCC AATAGTCGTT GAACCTAGTG TCAAAATGAC CTCTGTAACC
     1441 CTGC
//
```

Microinjection mixes:

Microinjection buffer (MIB; 10 mM Tris–HCl, 0.1 mM EDTA, 100 mM NaCl, pH7.5) was prepared and filtered through a 2 nm filter and autoclaved. Cas9 mRNA, sgRNAs and ssODNs were diluted and mixed in MIB to the working concentrations of 100 ng/µl, 50 ng/µl each

and 50 ng/µl, respectively. Injected embryos were re-implanted in CD1 pseudo-pregnant females. Host females were allowed to litter and rear F_0 progeny.

Sequence details

WT

GTTTCGTAGGGTGACTACACTGATAGACAACTAGAGGAACCTCTAGTCTTAATCTTTTTACAAATCTT
TTTTTTCCTTTGTTTTTATTTATTTATTATATGTAGGTACACTGCGGCTGTCCTCAGACACTCCAGAAG
AGGGAGTCAGATCTCATTATGGATGGTTGTGAGCCACCATGTGGTTGCTGGGATTTGAACTCCTGAC
CTTCGGAAGAACAGTCGGGTGCTCTTACCCACTGAGCCATCTCACCAGCCCCTCTAGTCTTAATCTTA
AAGATGTTGGAATATTAGTAATCCAACATCTTTAGTCATTGGTCATTGACTTTAGTCATTGGGTTCTA
TGCTGTCTGTGGAGCCTTTGAAGGCAGAAGACTATTTCCCAGCCTGTACTCATTTATATACACATAAA
TCTTTACATATACATTCTAGGTCTTTCCACTCTAGAATTGCCACAGACAACTCAATAACTCCTATAATG
GAGGCTTCTGAGCAAGGCTTTTATATTTCCTTTCAAGCACAGGCCCTTCTGAGGGGGCAGAGAAACT
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ATTCCAGCAGTAGAGAGATAGAGGCAGGAGAATCAGGAATTCATGATCATCTTTAGATATATAAAG
CATGTTCAAGGTCATTCTGACCCGGTCACACACACACACAAACAAACAAACAAACAAAAAAATCCCC
TTGGGCTTATAAAAACAGAAGGCATATTAATACATAACTTAGGACTTAGAACAAAATCATGACTCCC
ATCATGCTAACAAGCGCCCTGTATTCTGTTCCAG**AAGTTTCCAGAAGTGCATATGGAAGTCATTGCT**
GACTATGAGGTACACCCCAATCGGCGACCTAAGATTCTCGCCCAGACAGCAGCCCATGTGGCAGG
TGCTGCTTATTACTACCAACGACAAGATGTGGATGCAGACCCATGGGGGACCCAGGTTAGAGAGT
GAATGTGAATGAGTGGGTGGGGACCAAGGGAAAGGATAGTAAAGGCTCCTAAAGGATCCCCTACA
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GTTTTTAGATTGACACCCTCTGCTGGCCGCTGTGGGTACTGCTTGCATGCAAGCAAAACACTCATACC
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GGACTTACCGTGATGCTGTGACTCCTGAAGAACGGTACTCCGAAGAACAGAAGATCTACTTTTCCAC
CCCACCTGCCCAACGCTTGGCCCTATTAGGCTT

MMACHC-FLOX-EM1-B6

GTTTCGTAGGGTGACTACACTGATAGACAACTAGAGGAACCTCTAGTCTTAATCTTTTTACAAATCTT
TTTTTTCCTTTGTTTTTATTTATTTATTATATGTAGGTACACTGCGGCTGTCCTCAGACACTCCAGAAG
AGGGAGTCAGATCTCATTATGGATGGTTGTGAGCCACCATGTGGTTGCTGGGATTTGAACTCCTGAC
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CAG**AAGTTTCCAGAAGTGCATATGGAAGTCATTGCTGACTATGAGGTACACCCCAATCGGCGACCT**
AAGATTCTCGCCCAGACAGCAGCCCATGTGGCAGGTGCTGCTTATTACTACCAACGACAAGATGTG
GATGCAGACCCATGGGGGACCCAGGTTAGAGAGTGAATGTGAATGAGTGGGTGGGGACCAAGGG
AAAGGATAGTAAAGGCTCCTAAAGGATCCCCTACAATATAAGGTCTGTATAAGTGCTGAGATTAAA
GGTGTGCCCCACCACTGCCCGGCTAGATTTTTTTTT**[1nt_del]**AAAGATTTATTTATTTATTGTATGTA
AGTACACTGTAGCTATCTTCAGACACACCAGAAGAGGGCATCAGTTCTCGTTACAGATGGTTGTGAG
CCACCATGTGGTTGCTGGGATTTGAACTCAGGACCTTCAGAAGAGCAGTCAGTGCTCTTCACCACTG
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GGGGCTGGTTTGCCATCCGAGGGGTTATGTTGCTGCCAGGGATTGAAGTGCCAAATTTGCCACCCA
GAAAGCCCCCTGACTGTGTGCCTACAAGAGCTGGCCGCATCACTCTGCTTGAAGGTTTCAATTTCCAT
TGGCGGGACTGGACTTACCGTGATGCTGTGACTCCTGAAGAACGGTACTCCGAAGAACAGAAGATC
TACTTTTCCACCCCACCTGCCCAACGCTTGGCCCTATTAGGCTT

LoxP sites are highlighted in red and genotyping handles (restriction enzyme site plus primer unique to each LoxP site) in yellow. Exons are highlighted in grey, with floxed exon in bold also. Blue highlights a 1 nt deletion in the floxed region.

Nucleotide Alignment:

LoxP sites are highlighted in red and genotyping handles (restriction enzyme site plus primer unique to each LoxP site) in yellow. Exons are highlighted in grey. The 1 nt deletion in the critical region is highlighted in light blue.

QC strategy employed at Harwell to check the edited allele:

Genomic DNA was extracted from ear clip biopsies and amplified in a PCR reaction using the following conditions/primer sequences:

Geno_Mmachc_F1 primer (5'-3')	GTTTCGTAGGGTGACTACACTGA
Geno_Mmachc_R1 primer (5'-3')	AAGCCTAATAGGGCCAAGCG
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	64
Elongation time (min)	1
WT product size (bp)	1917
Mutant product size (bp)	2000
Notes	Also sequenced with LoxPF and LoxPR primers as detailed below.

LoxPF (5'-3')	atccgggggtaccgcgtcgag
LoxPR (5'-3')	actgatggcgagctcagacc
Taq Polymerase used	Roche Expand Long Range DNTPack
Annealing Temperature (°C)	61
Elongation time (min)	3 m 10 s
WT product size (bp)	N/A
Mutant product size (bp)	1007
Notes	PCR used to screen for floxed alleles. 3% DMSO used in reactions.

Geno_Mmachc_F1 primer (5'-3')	GTTTCGTAGGGTGACTACACTGA
LoxPR (5'-3')	actgatggcgagctcagacc
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60

Elongation time (min)	1
WT product size (bp)	N/A
Mutant product size (bp)	1461
Notes	PCR indicates whether donor integrated on target.

LoxPF (5’-3’)	atccgggggtaccgcgtcgag
Geno_Mmachc_R1 primer (5’-3’)	AAGCCTAATAGGGCCAAGCG
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60
Elongation time (min)	1
WT product size (bp)	N/A
Mutant product size (bp)	1546
Notes	PCR indicates whether donor integrated on target.

Amplicons were sent for Sanger sequencing to check for integration of the donor oligo sequence at the target site. F1 sequences should be heterozygous unless on sex chromosome.

The potential 1 nt deletion in the floxed region was confirmed by sequencing amplicons from the following PCR:

Geno_Mmachc_F5 primer (5’-3’)	GAGGGGGCAGAGAAACTCTAAA
Geno_Mmachc_R5 primer (5’-3’)	GAAGGCTCTGTCCCTTCT
Taq Polymerase used	ThermoFisher SuperFi II PCR Kit
Annealing Temperature (°C)	60
Elongation time (min)	0.5
WT product size (bp)	829
Mutant product size (bp)	829
Notes	

Off-target site with ≤2 mismatches in the seed sequence of guide(s) used were checked with the following primers:

Off-target site	Sequence	Type	Primers used (5'-3')
1:9291611-9291633	AGGAGTTATTGAGTTGT**GC**G AGG	Intronic	Geno_Mmachc_OT1F1: TCACAGTTTTAGCAGAACCTCCA Geno_Mmachc_OT1R1: TGTGTAAATGTTTGTGGGAGCTG
5:111286563-111286585	GG**CTG**GAGTGTGCACTGAGT TGG	Exonic	Geno_Mmachc_OT2F1: TCAGCCTGCTGAACTTGTCC Geno_Mmachc_OT2R1: CCTGTGGCAGGAGCATAACT
4:107892511-107892533	GGA**GCA**AGTG**A**GCACTGAGT TGG	Intronic	Geno_Mmachc_OT3F1: TCCTGCTGTAGTGTCAGGGA Geno_Mmachc_OT3R1: ACCATGTCAGGCTGGAGAGA
13:51701429-51701451	G**A**ACACTCATTCCATG**T**AGA GGG	Exonic	Geno_Mmachc_OT4F1: CAAGATCCCAGGACCAACCC Geno_Mmachc_OT4R1: CTTGGACTTCCGCCTTGACT
12:93036538-93036560	GC**CA**ACTC**C**TTCCATGCAGA TGG	Intergenic	Geno_Mmachc_OT5F1: ATTCTGTGGGCTGAGATGCT Geno_Mmachc_OT5R1: TGGCTCATTTGGGGATCCTT Sequenced with: Geno_Mmachc_OT5F2: GTTCTTCCTATGGGGTTTGAA Geno_Mmachc_OT5R2: AATGAGAAGGTCTGCTAGAG

All amplicons were sent for Sanger sequencing. No evidence of off-target effects was observed in those animals selected for breeding.

Additional integrations of the donor sequence

Copy counting of the donor sequence was carried out by ddPCR at the F1 stage to confirm donor oligos were inserted once on target into the genome. The following Taqman assay was used to copy count the donor sequence compared against a VIC-labelled reference assay for Dot1l:

Assay name	MMACHC-5'-FLOX-MUT1
Forward Primer (5'-3')	ACATTCTAGGTCTTTCCACTC
Reverse Primer (5'-3')	AGCCTCCAATAACTTCGTATAA
Probe (5'-3')	TCGAGGCGATCGCATAACTTCG
Label	FAM

This ddPCR assay is specific to the Mmachc flox donor and only floxed alleles are expected to be recognised by this assay. WT controls are expected to call at 0 copies and a single integration for a correct mutation is expected to call at 1 copy for F1 (HET) animals.

Assay name	MMACHC-CR-LOA-WT1
Forward Primer (5'-3')	TTGCTGACTATGAGGTACAC
Reverse Primer (5'-3')	CATCTTGTCGTTGGTAGTAATAAG
Probe (5'-3')	CGACCTAAGATTCTCGCCCAGACA
Label	FAM

This ddPCR assay is universal to Mmachc - both WT and floxed alleles are recognised by this assay. WT controls are expected to call at 2 copies and a single integration for a correct mutation is expected to call at 2 copies for F1 (HET) animals.

Assay name	MMACHC-3'-FLOX-MUT1
Forward Primer (5'-3')	GGGACAGAGCCTTCTATAAC
Reverse Primer (5'-3')	TTCTTTCCCACTACTGATGG
Probe (5'-3')	AAGTTATCGCCGGCGGGTCTGA
Label	FAM

This ddPCR assay is specific to the Mmachc flox donor and only floxed alleles are expected to be recognised by this assay. WT controls are expected to call at 0 copies and a single integration for a correct mutation is expected to call at 1 copy for F1 (HET) animals.

Reference Assay Name	Dot1l
Forward primer (5’-3’)	GCCCCAGCACGACCATT
Reverse primer (5’-3’)	TAGTTGGCATCCTTATGCTTCATC
Probe (5’-3’)	CCCAACAGGCCTGGATTCTCAATGC
Label	VIC

VIC-labelled reference assay for Dot1l gene.

No evidence of additional donor integrations was detected in the animals taken forward to establish the colony.

MMACHC-FLOX-EM1

Allele Description

This is a CRISPR/Cas9 induced mutation creating a conditional knock-out by floxing critical exon, ENSMUSE00000181372 of *Mmachc*. The stock was generated at MRC Harwell via pronuclear injection of CRISPR/Cas9 reagents into 1-cell stage embryos.

qPCR Copy Counting Genotyping Strategy

The genotyping strategy presented here has been optimized for reagents and conditions used by the Genotyping Core at MRC Harwell. To genotype animals, we recommend researchers validate the assay independently. PCR cycling temperature and times may require additional optimization based on the specific genotyping reagents used.

Samples are genotyped using qPCR copy counting with both a wildtype loss of allele (WT-LOA) and a mutant assay against a known reference assay (*Dot1l* on chromosome 10; 2 copies present). Samples for this line are genotyped using the following primers and probe:

- Universal probe and Universal primer designed 5' of the deleted region.
- Wildtype specific primer situated within the deleted region.
- Mutant specific primer that binds to the inserted LoxP sequence

For autosomal genes that have been targeted, the following results would be expected:

Genotype of the Modified allele	WT Assay	Mutant Assay
Wildtype	2	0
Heterozygous	1	1
Homozygous mutant	0	2

MMACHC-FLOX-3'-WT1 assay (FAM labelled)

GAGCAGTCAGTGCTCTTCACCACTGAG**CCATCTCTCCAGCCCTAGATTT**CTTAATTTAATAGAAGGG
ACAGA**GCCTTCTGCATGGAATGAGTGTGCA**CTGAGTGGGAAAGAATTAGAAAAAGATTAGGAACA
TTTGTTGCTTTTGTATGAACC**ATGTTCAATCCCAAGCACTCAC**GTGGCTGCTCAGAACCATCTGCAAC

Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo MMACHC-FLOX	5' label	Sequence 5' → 3'	3' label	Oligo Type
MMACHC-FLOX-WT_F	n/a	**CCATCTCTCCAGCCCTAGATTT**	n/a	WT Forward
MMACHC-FLOX-WT_PROBE	FAM	**TGCACACTCATTCCATGCAGAAGGC**	ZEN-IBFQ	WT Probe
MMACHC-FLOX-WT_R	n/a	**GTGAGTGCTTGGGATTGAACAT**	n/a	WT Reverse

MMACHC-FLOX-3'-MUT1 assay (FAM labelled)

CCATGTGGTTGCTGGGATTTGAACTCAGGACCTTCAGAAGAGCAGTCAGTGCTCTTCACCACTGAGC
CATCTCTCCAGCCCTAGATTTCTTAATTTAATAGAA**GGGACAGAGCCTTCTATAAC**TTCGTATAGCAT
ACATTATACG**AAGTTATCGCCGGCGggtctga**gctcg**ccatcagtAGTGGGAAAGAA**TTAGAAAAAGATT
AGGAACATTTGTTGCTTTTGTATGAACCATGTTCAATCCCAAGCACTCACGTGGCTGCTCAGAACCAT

Lower case letters denote the inserted LoxP sequence
Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo MMACHC-FLOX	5' label	Sequence 5' → 3'	3' label	Oligo Type
MMACHC-FLOX- MUT_F	n/a	**GGGACAGAGCCTTCTATAAC**	n/a	Mutant Forward
MMACHC-FLOX-MUT_PROBE	FAM	**AAGTTATCGCCGGCGGGTCTGA**	ZEN-IBFQ	Mutant Probe
MMACHC-FLOX-MUT_R	n/a	**TTCTTTCCCACTACTGATGG**	n/a	Mutant Reverse

Dot1l internal control (VIC labelled)

CTGATGGGTGTGGGCAGATCCTACAGAGTCCCATTGGCCACCATGTGTGCTACGCCTGAAATAAAGCCTT**GCC CCAGCACGACCATT**CAGGG**CCAGCTCTCAAGTCG**ACTGTAA**GATGAAGCATAAGGATGCCAACTA**CTAACA GAAAACGACTAGAGGGGAAAAGAACAAGGAAACAGAAGACGCAGCACTCCGGCTTCCCTGGGTTGGCCAGT CACCCTATGA

Oligo MMACHC-FLOX	5' label	Sequence 5' → 3'	3' label	Oligo Type
Dot1l_Forward	n/a	**GCCCCAGCACGACCATT**	n/a	WT Forward
Dot1l_Probe	VIC	**CCAGCTCTCAAGTCG**	BHQ	WT Probe
Dot1l_Reverse	n/a	**TAGTTGGCATCCTTATGCTTCATC**	n/a	WT Reverse

Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

DNA extraction method

DNA is extracted from ear clips using Applied Biosystems Taqman Sample-to-SNP Kit and qPCR run using 1:10 dilution from the crude preparation.

qPCR master mix 1X

Applied Biosystems GTX Taqman master mix	5 µl
Dot1l_Forward (20 µM)	0.225 µl
Dot1l_Reverse (20 µM)	0.225 µl
Dot1l_Probe (5 µM)	0.2 µl
FAM Assay (probe 5 µM & primers 15 µM each)	0.3 µl
ddH20	1.55 µl
DNA (1:10 dilution of ABI Sample-to-SNP prep)	2.5 µl

Each sample is ran in technical duplicate. Seven WT and/or mutant controls are also included in duplicate along with non-template controls.

qPCR cycling conditions

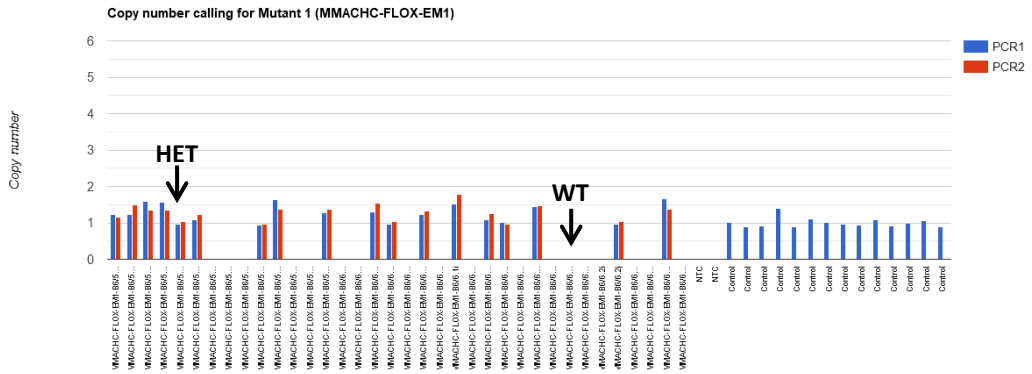
qPCR instrument: Applied Biosystems 7500/7900 or ThermoFisher QuantStudio 7

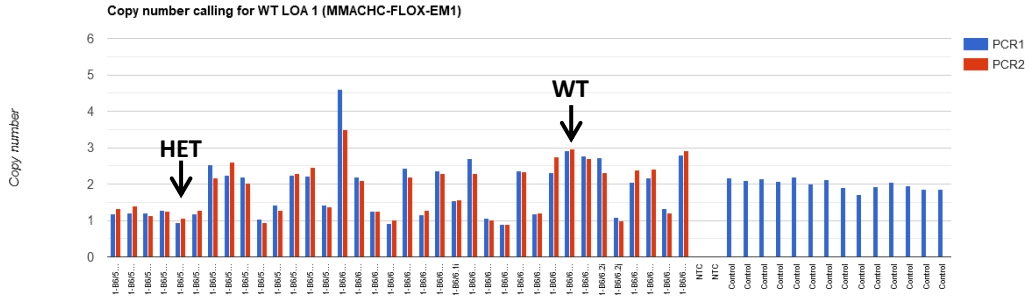
95°C for 20 sec
Then 40 cycles of;
95°C for 3 sec
60°C for 30 sec

Analysis

The results are analysed using CopyCaller software v2.0 from Applied Biosystems or in-house software that is based on CopyCaller v2.0.

MMACHC-FLOX'-WT1 and MMACHC-FLOX-MUT1 assays copy called results, image showing copy number chart for WT and Mutant assays (Task 347633 results)





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Created/Updated by:	Daniel Ford
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